Translation of claims only

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[Title of the Invention]

CHROMATOGRAPHIC TECHNIQUE AND CHROMATOGRAPHIC PACKING TO BE USED THEREIN

[Abstract]

[Object] To provide a chromatographic technique with the use of a chromatographic packing by which a biological factor (protein, DNA, glycolipid, etc.) or a cell can be separated or purified by controlling its interaction with the surface of a solid in an aqueous system by an external signal (for example, temperature).

[Constitution]

A chromatographic technique for separating a solute by using a packing wherein the hydrophilic-hydrophobic balance on the surface of a stationary phase can be changed by an external signal while fixing the mobile phase to an aqueous system. More particularly, a chromatographic technique with the use of a chromatographic packing wherein the surface of a carrier having amino groups, carboxyl groups, hydroxyl groups or the like on the surface is chemically modified with a polyalkylacrylamide having a terminal amino, carboxyl or hydroxyl group or the like or a copolymer of the same.

[Claims]

[Claim 1] A chromatographic technique characterized in that a solute is separated by using a packing wherein the hydrophilic-hydrophobic balance on the surface of a stationary phase can be changed by an external signal while fixing the mobile phase to an aqueous system.

[Claim 2] A chromatographic technique as claimed in Claim 1 wherein said external signal is a change in temperature.

[Claim 3] A chromatographic technique as claimed in Claim 1 wherein said solute is a biological factor or a cell.

[Claim 4] A chromatographic technique as claimed in Claim 1 wherein said packing is a chromatographic packing wherein the surface of a carrier is chemically modified with a polyalkylacrylamide having a terminal amino, carboxyl or hydroxyl group or the like or a copolymer of the same.

[Claim 5] A chromatographic technique which comprises retaining a solute by a stationary phase comprising a chromatographic packing wherein a carrier having amino groups, carboxyl groups, hydroxyl groups or the like on the surface is chemically modified with a polyalkylacrylamide having a terminal amino, carboxyl or hydroxyl group or the like or a copolymer of the same; and allowing the solute to pass through a single mobile phase while changing the hydrophilic-hydrophobic balance on the surface of the stationary phase by the temperature gradient method or the temperature-step

gradient method wherein the external temperature is varied stepwise to thereby separate the solute.

[Claim 6] A chromatographic technique as claimed in Claim 5 wherein said mobile phase is an aqueous solvent.

[Claim 7] A chromatographic packing wherein a temperature responsive polymer has been introduced onto the surface of a carrier.

[Claim 8] A chromatographic packing as claimed in Claim 7 wherein said temperature responsive polymer is a polyalkylacrylamide having a terminal amino, carboxyl or hydroxyl group or the like.

[Claim 9] A chromatographic packing as claimed in Claim 8 wherein said polyalkylacrylamide is one selected from among poly(N-isopropylacrylamide), polydiethylacrylamide and polyacryloylpyrrolidine.

[Claim 10] A chromatographic packing wherein the surface of a carrier having amino groups, carboxyl groups, hydroxyl groups or the like is chemically modified with a polyalkylacrylamide having an amino, carboxyl or hydroxyl group or the like or a copolymer of the same.

[Detailed Description of the Invention]

[0001]

[Field of Industrial Application]

This invention relates to a chromatographic technique with the use of a chromatographic packing by which a

biological factor (protein, DNA, glycolipid, etc.) or a cell can be separated or purified by controlling the interaction between the surface of a solid and cell membrane in an aqueous system by an external signal (for example, temperature).

[0002]

In recent years, high performance liquid chromatography (HPLC) has been employed in the separation and purification of many substances, since various combinations of mobile phases and stationary phases can be appropriately selected therein depending on the sample. In the conventional chromatographic techniques, however, the interaction between a solute contained in the mobile phase and the surface of the stationary phase is induced by changing not the surface structure of the stationary phase but a solvent in the mobile phase. In HPLC employed in a number of fields, for example, carriers such as silica gel are employed as the stationary phase while organic solvents such as hexane, acetonitrile and chloroform are employed as the mobile phase in the case of normal phase columns. In the case of reversed phase columns wherein silica gel derivatives separated in an aqueous system are employed as carriers, on the other hand, use is made of organic solvents such as methanol and acetonitrile.

[0003]

In ion exchange chromatography with the use of anion exchangers or cation exchangers as the stationary phase,

substances are separated by changing the external ion concentration or ion type. With the recent rapid progress in genetic engineering, etc., it has been expected to use physiologically active peptides, proteins, DNAs and the like in various fields including the pharmaceutical field. Thus it is a very important problem to separate and purify these substances. Among all, there has been a great increase in the necessity for techniques for separating and purifying physiologically active substances without damaging the activities thereof.

[0004]

However, the organic solvents, acids, alkalis and surfactants employed in the conventional mobile phases would damage the activities of physiologically active substances and, moreover, contaminate the same. It is therefore expected to improve this system. From the viewpoint of avoiding environmental pollution due to these substances, it has been also required to establish a separation and purification system without using these substances.

[0005]

[Problems to be Solved by the Invention]

Under these circumstances, the present inventors have conducted extensive studies to satisfy the above-mentioned requirements. As a result, they have successfully developed a technique wherein separation and purification are achieved by

changing the interaction between a solute and the stationary phase surface by changing not the mobile phase but an external condition such as temperature, thus completing the present invention. Accordingly, the present invention aims at providing a chromatographic technique wherein separation and purification can be achieved with the use of a mobile phase of a single aqueous system by reversibly changing the surface characteristics of the stationary phase due to changes in an external condition, and a packing which is to be used as the stationary phase in this chromatographic technique.

[0006]

[Means for Solving the Problems]

The gist of the present invention resides in a chromatographic technique for separating a solute by using a packing wherein the hydrophilic-hydrophobic balance on the surface of a stationary phase can be changed by an external signal while fixing the mobile phase to an aqueous system, more particularly, a chromatographic technique with the use of a chromatographic packing wherein the surface of a carrier having amino groups, carboxyl groups, hydroxyl groups or the like on the surface is chemically modified with a polyalkylacrylamide having a terminal amino, carboxyl or hydroxyl group or the like or a copolymer of the same. According to the present invention, namely, a biological factor such as a protein or cell is adsorbed onto hydrophobic

surface by elevating the external temperature to the critical temperature or above and then the temperature is lowered. Thus the biological factor can be separated or peeled off. Since no chemical (organic solvent, acid, alkali, surfactant, etc.) is employed in this process, the chromatographic system can be prevented from the contamination with these chemicals. Moreover, this technique is applicable to separation similar to analysis while sustaining the function of the protein or cell.

[0007]

By the conventional chromatographic techniques, it is highly difficult to separate and analyze samples containing various compounds, in particular, two or more samples largely differing from each other in polarity in a single mobile phase. Thus it takes a very long period of time to complete the separation. To deal with such samples, therefore, it has been a practice to employ the solvent gradient method or the step gradient method wherein the amount or type of organic solvent(s) is continuously varied with the passage of time to thereby separate the solute. In the temperature gradient method or the step gradient method of the present invention, in contrast, separation can be similarly achieved by continuously or stepwise varying the column temperature in a single mobile phase without using any organic solvent. By using such a method, it becomes possible to separate proteins,

cells, etc. while sustaining the functions thereof and preventing the contamination with the above-mentioned impurities. Moreover, the desired component can be separated within a short period of time by controlling the temperature.

[0008]

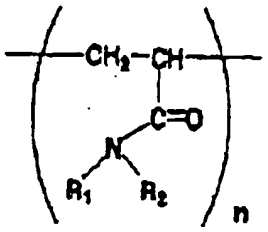
The chromatographic packing to be used in the present invention is one having a temperature responsive polymer introduced onto the surface thereof. Thus the hydrophilic-hydrophobic balance on the surface of the packing can be changed depending on, for example, temperature changes. In this packing, therefore, the carrier surface is chemically modified with a temperature responsive polymer, for example, a polyalkylacrylamide having a terminal amino, carboxyl or hydroxyl group or the like or a copolymer thereof. Examples of this chemically modified packing include those obtained by chemically modifying silica carriers having functional groups such as amino, carboxyl or hydroxyl groups on the surface with the above-mentioned polyalkylacrylamides or copolymers thereof. Particular examples of the silica carriers having functional groups such as amino, carboxyl or hydroxyl groups include aminopropyl silica gel, Amino Sephadex, aminoglass and ion exchange resins. As the polyalkylacrylamide to be used in the present invention, it is preferable to select one from among poly(N-isopropylacrylamide), polydiethyleneacrylamide and polyacryloylpyrrolidine. That is to say, the structural

formulae of the polyalkylacrylamide or its polymer preferably employed in the present invention are as follows:

[0009]

[Chemical formula 1]

Polyalkylacrylamide

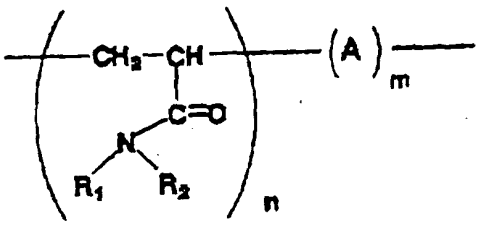


	R_1	R_2	Abbreviation
Poly(*N*-isopropylacrylamide)	—H	—CH(CH$_3$)$_2$	Poly(IPAAm)
Poly(*N*,*N*'-diethylacrylamide)	—C_2H_5	—C_2H_5	Poly(DEAAm)
Poly(acryloylpyrolidine)			Poly(APy)

[0010]

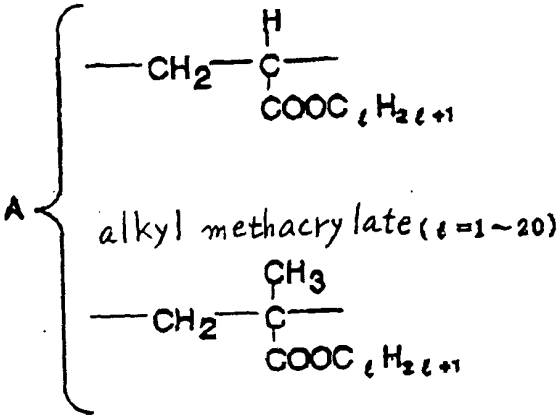
[Chemical formula 2]

Copolymer



A: content: 5–60%

alkyl acrylate (ℓ=1~20)



alkyl methacrylate (ℓ=1~20)

[0011]

Since poly(N-isopropylacrylamide) has a lower limit critical temperature of 32 °C, a carrier chemically modified with its molecules undergoes changes in the surface characteristics (hydrophilic/hydrophobic) at this critical temperature. When the surface of a chromatographic packing is grafted or coated therewith, therefore, the capability of retaining a sample varies depending on temperature. Thus the

retention behaviors can be controlled depending on temperature without changing the composition of the eluent. The lower limit critical temperature exceeding 32 °C can be achieved by copolymerizing isopropylacrylamide with a monomer superior in hydrophilic nature to it, for example, acrylamide, methacrylic acid, acrylic acid, dimethylacrylamide or vinylpyrrolidone. Also, a lower limit critical temperature of 32 °C or below can be achieved by copolymerizing isopropylacrylamide with a hydrophobic monomer such as styrene, alkyl methacrylate or alkyl acrylate.

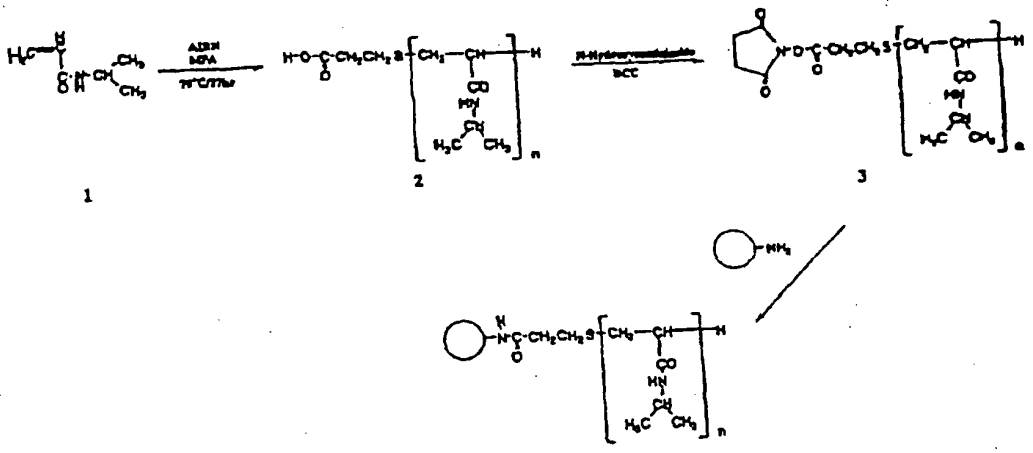
[0012]

Because of having a lower limit critical temperature of about 30 to 32 °C, polydiethylacrylamide undergoes changes in the surface characteristics (hydrophilic/hydrophobic) at this point. Thus its capability of retaining a sample can be controlled by varying the temperature, similar to the above-mentioned case of poly(N-isopropylacrylamide). The novel chromatographic packing employed in the present invention can be prepared by chemical modification or polymer coating. As a means of the chemical modification, use can be made of two methods, i.e., surface grafting and radical polymerization. In the coating method, the polymer is insolubilized at a temperature falling within the application range and then subjected to coating. Fig. 1 illustrates this method. Now, an example of the means for producing the chromatographic

packing of the present invention will be described by reference to the following chemical formula.

[0013]

[Chemical formula 3]



[0014]

N-Isopropylacrylamide monomer (1), 2,2'-azobis-(isobutyronitrile) (abbreviated as AIBN) and 3-mercapto-propionic acid (abbreviated as MPA) are dissolved in a solvent N,N-dimethylformamide. After freeze-degassing with the use of liquid nitrogen, these monomers are polymerized by telomerization at 70 ± 1 °C. Then the mixture is concentrated and precipitated from diethyl ether to thereby give poly(N-isopropylacrylamide) (2) having a terminal carboxyl group. The crude product is purified by dissolution-reprecipitation.

Then it is introduced into a desiccator containing silica gel and dried at ordinary temperatures under reduced pressure. Then it is dissolved in dry ethyl acetate and dicyclohexylcarbodiimide (abbreviated as DCC) and N-hydroxysuccinimide are added thereto. After reacting at room temperature to thereby convert the carboxyl group of the poly(N-isopropylacrylamide) into an active ester, it is concentrated and dropped into diethyl ether for precipitation. Next, it is dried at ordinary temperatures under reduced pressure to thereby give active-esterified poly(N-isopropylacrylamide) (3). The obtained product is dissolved in purified water and a carrier having amino groups is added thereto. Then these substances are reacted together to thereby form an amide bond. Thus a carrier (4) graft-coated with poly(N-isopropylacrylamide) is obtained. By using the carrier of the present invention, physiologically active proteins, cells, etc. can be separated and purified. Particular examples thereof include bovine serum albumin, IgG, fibrinogen, fibronectin, transferrin and blood coagulation factor.

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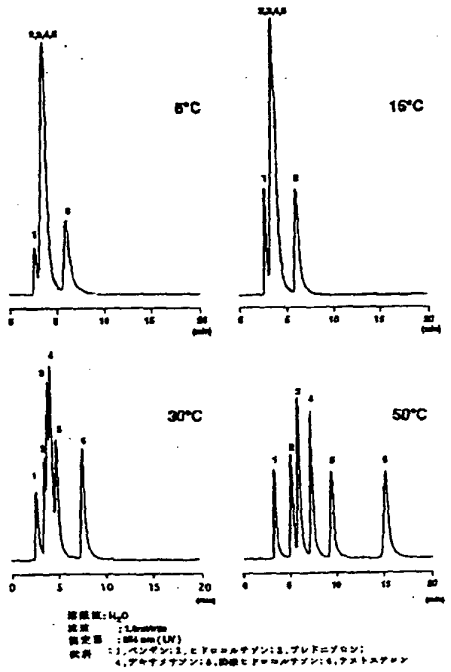
最終頁に続く

(54)【発明の名称】　クロマトグラフィー方法及び該方法に使用するクロマトグラフィー用充填剤

(57)【要約】
【目的】水系で生体要素（タンパク質、ＤＮＡ、糖脂質等）及び細胞を固体表面との相互作用を外的信号（例えば温度）によって制御し、分離あるいは精製することができるクロマトグラフィー用充填剤を使用したクロマトグラフィー方法提供することを目的とする。
【構成】移動相を水系に固定したままで、固定相表面の親水性／疎水性のバランスを外的信号によって変化させることが可能である充填剤を用いて溶質の分離を行うことを特徴とするクロマトグラフィー方法であり、具体的には、アミノ基、カルボキシル基、或いは水酸基等を表面に持つ担体表面を、末端にアミノ基、カルボキシル基、或いは水酸基等を有するポリアルキルアクリルアミド或いはその共重合体で化学修飾したクロマトグラフィー用充填剤を用いたクロマトグラフィー方法である。



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【特許請求の範囲】

【請求項１】　移動相を水系に固定したままで、固定相表面の親水性／疎水性のバランスを外的信号によって変化させることが可能である充填剤を用いて溶質の分離を行うことを特徴とするクロマトグラフィー方法。

【請求項２】　外的信号が温度変化である請求項１記載のクロマトグラフィー方法。

【請求項３】　溶質が生体要素もしくは細胞である請求項１記載のクロマトグラフィー方法。

【請求項４】　充填剤が、担体表面を末端にアミノ基、カルボキシル基、或いは水酸基等を有するポリアルキルアクリルアミド或いはその共重合体で化学修飾したクロマトグラフィー用充填剤である請求項１記載のクロマトグラフィー方法。

【請求項５】　アミノ基、カルボキシル基、或いは水酸基等を表面に持つ担体に、末端にアミノ基、カルボキシル基、或いは水酸基等を有するポリアルキルアクリルアミド或いはその共重合体で化学修飾したクロマトグラフィー用充填剤よりなる固定相に溶質を保持させた後、外部温度を段階的に変化させる温度グラディエント或いは温度によるステップグラディエント法により固定相表面の親水性／疎水性のバランスを変化させ、同一の移動相を通過させることによって溶質を分離することを特徴とするクロマトグラフィ方法。

【請求項６】　移動相が水系溶媒である請求項５記載のクロマトグラフィー方法。

【請求項７】　担体表面に、温度応答性高分子を導入したことを特徴とするクロマトグラフィー用充填剤。

【請求項８】　温度応答性高分子が末端にアミノ基、カルボキシル基、或いは水酸基等を有するポリアルキルアクリルアミドである請求項７記載のクロマトグラフィー用充填剤。

【請求項９】　ポリアルキルアクリルアミドが、ポリー（Ｎーイソプロピルアクリルアミド）、ポリジエチルアクリルアミド又はポリアクリロイルピロイジンの何れか一種である請求項８記載のクロマトグラフィー用充填剤。

【請求項１０】　アミノ基、カルボキシル基、或いは水酸基等を有する担体表面にアミノ基、カルボキシル基、或いは水酸基等を有するポリアルキルアクリルアミド或いはその共重合体を化学修飾したことを特徴とするクロマトグラフィー用充填剤。

【発明の詳細な説明】

【０００１】

【産業上の利用分野】本発明は水系で、生体要素（タンパク質、ＤＮＡ、糖脂質等）及び細胞を固体表面と細胞膜との相互作用を外的信号（例えば温度）によって制御することが可能であるクロマトグラフィー用充填剤を利用して分離或いは精製することができるクロマトグラフィー方法に関する。

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【０００２】

【従来の技術】高速液体クロマトグラフィー（ＨＰＬＣ）は移動相液体と固定相の組合せが多種多様であり、試料に応じて種々選択できるので近年種々の物質の分離、精製に利用されている。しかして、従来使用されているクロマトグラフィーでは固定相の表面構造は変化させずに、移動相中に含まれている溶質と固定相表面との相互作用を移動相の溶媒を変化させることによって行われている。例えば、多くの分野で使用されているＨＰＬＣにおいては、固定相としてシリカゲル等の担体を用いた順相系のカラムではヘキサン、アセトニトリル、クロロホルムなどの有機溶媒を移動相として使用しており、また水系で分離されるシリカゲル誘導体を担体として用いた逆相系のカラムではメタノール、アセトニトリルなどの有機溶媒が使用されている。

【０００３】また、陰イオン交換体あるいは陽イオン交換体を固定相とするイオン交換クロマトグラフィーでは外的イオン濃度あるいは種類を変化させて物質分離を行っている。近年遺伝子工学等の急速な進歩により、生理活性ペプチド、タンパク質、ＤＮＡなどが医薬品を含む様々な分野に広範囲にその利用が期待され、その分離・精製は極めて重要な課題となっている。特に、生理活性物質をその活性を損なうことなく分離・精製する技術の必要性が増大している。

【０００４】しかし、従来の移動相に用いられている有機溶媒、酸、アルカリ、界面活性剤は生理活性物質の活性を損なうと同時に夾雑物となるために、そのシステムの改良が期待されている。また、このような物質の環境汚染の回避という面からもこれらの物質を用いない分離・精製システムが必要となっている。

【０００５】

【発明が解決しようとする課題】そこで、本発明者らは、上記の要望を満足すべく種々検討した結果、固定相の表面構造を、例えば温度などの外的条件を変化させることによって、移動相を変化させることなく溶質と固定相表面との相互作用を変化させることにより分離・精製する技術を開発し、本発明を完成したもので、本発明の目的は、外的条件を変化させることによって固定相の表面特性を可逆的に変化させ、これによって単一の水系移動相によって分離、精製可能なクロマトグラフィー方法及び該クロマトグラフィーに使用する固定相としての充填剤を提供する。

【０００６】

【課題を解決するための手段】本発明の要旨は、移動相を水系に固定したままで、固定相表面の親水性／疎水性のバランスを外的信号によって変化させることが可能である充填剤を用いて溶質の分離を行うことを特徴とするクロマトグラフィー方法であり、具体的には、アミノ基、カルボキシル基、或いは水酸基等を表面に持つ担体表面を、末端にアミノ基、カルボキシル基、或いは水酸

基等を有するポリアルキルアクリルアミド或いはその共重合体で化学修飾したクロマトグラフィー用充填剤を用いたクロマトグラフィー方法である。即ち、本発明を用いることにより、外部温度を臨界温度以上にすることによってタンパク質や細胞などの生体要素を疎水性表面に吸着させ、温度を低下させることにより、これを分離又は剥離することが可能となる。従って、この際、有機溶媒、酸、アルカリ、界面活性剤等の薬剤を全く用いないので、これらが夾雑物質となることを防ぎ、また、タンパク質や細胞などの機能を維持したままでの分析と同じに分離にも利用することができる。

【０００７】従来のクロマトグラフィー法では１種類の移動相で種々の化合物が混じっている試料特に極性の大きく異なる複数の試料を分離・分析する場合、分離が困難であり、分離に要する時間が大変長くなってしまう。そのため、このような試料を扱う際には有機溶媒の量や種類を時間と共に連続的に変化させる溶媒グラディエント法或いは段階的に変化させるステップグラディエント法により分離を行っているが、本発明による温度グラディエント法或いはステップグラディエント法では有機溶媒を使用する代わりに単一の移動相でカラム温度を連続的或いは段階的に変化させることにより同様の分離を達成することが可能であり、この方法を採用することによって、上述の夾雑物の混入を防止し、タンパク質や細胞などの機能を維持したままで分離できると共に所望の成分を温度をコントロールすることによって短時間で分離＊

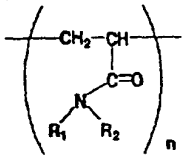
＊が可能なのである。

【０００８】本発明において使用するクロマトグラフィー用充填剤は、その表面に温度応答性高分子を導入し、これによって充填剤表面の親水性／疎水性のバランスが、例えば温度変化によって変化することが可能な充填剤である。即ち、担体表面を温度応答性高分子である、例えば末端にアミノ基、カルボキシル基、或いは水酸基等を有するポリアルキルアクリルアミド或いはその共重合体で化学修飾した充填剤である。この化学修飾した充填剤としては、例えば、表面にアミノ基、カルボキシル基、或いは水酸基等の官能基を有するシリカ担体に前記のポリアルキルアクリルアミド或いはその共重合体を化学修飾したものである。そして、アミノ基、カルボキシル基、或いは水酸基等の官能基を有するシリカ担体としては、具体的にアミノプロピルシリカゲル、アミノセファデックス、アミノガラス、イオン交換樹脂等を挙げることができる。本発明において、ポリアルキルアクリルアミドとしては、ポリー（Ｎ－イソプロピルアクリルアミド）、ポリジエチルアクリルアミド又はポリアクリロイルピロリジンの何れか一種が好ましい。従って、本願発明において使用する好ましいポリアルキルアクリルアミド及びその共重合体の構造式を示すと次の通りである。

【０００９】

【化１】

ポリーアルキルアクリルアミド



	R_1	R_2	Abbreviation
Poly(*N*-isopropylacrylamide)	—H	—CH(CH₃)₂	Poly(IPAAm)
Poly(*N*,*N*'-diethylacrylamide)	—C_2H_5	—C_2H_5	Poly(DEAAm)
Poly(acryloylpyrolidine)			Poly(APy)

【００１０】

【化２】

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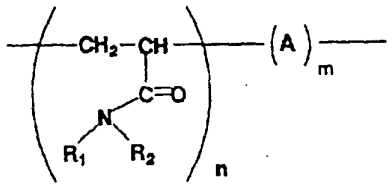
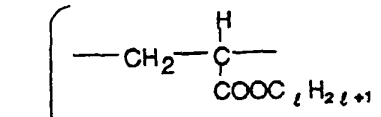
共重合体

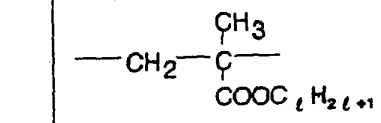


A：5～60%含有

アルキルアクリレート（ℓ=1～20）



アルキルメタクリレート（ℓ=1～20）



【0011】ポリ（N－イソプロピルアクリルアミド）は32℃に下限臨界温度を有するので、該分子で化学修飾した担体はこの臨界温度で親水／疎水に表面物性が変化するため、これをクロマトグラフィーの充填剤の表面にグラフトもしくはコーティングして使用した場合、試料に対する保持力が温度によって変化するため溶離液の＊

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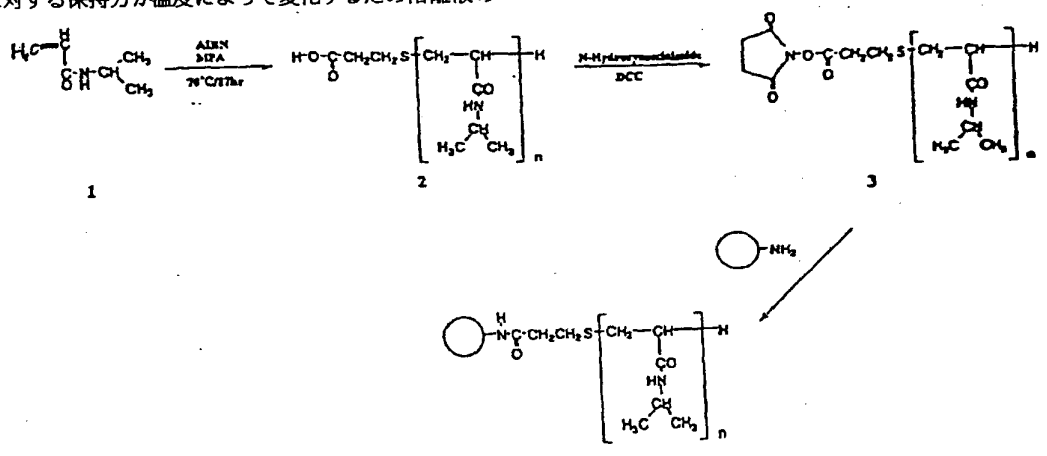
＊組成を変化させずに保持挙動を温度によってコントロールすることが可能となる。下限臨界温度を32℃以上にするためには、イソプロピルアクリルアミドよりも親水性のモノマーであるアクリルアミド、メタアクリル酸、アクリル酸、ジメチルアクリルアミド、ビニルピロリドンなどをイソプロピルアクリルアミドと共重合させることによって調整することが可能である。また、下限臨界温度を32℃以下にしたいときは、疎水性モノマーであるスチレン、アルキルメタクリレート、アルキルアクリレートなどとの共重合によって調整することができる。

【0012】また、ポリジエチルアクリルアミドの下限臨界温度は、約30℃～32℃であり、この温度を境として親水／疎水に表面物性が変化し、前述のポリー（N－イソプロピルアクリルアミド）の場合と同様に、試料に対する保持力が温度によって調整することができる。本発明で利用される新規なクロマトグラフィー用担体は、化学修飾或いは高分子のコーティングによって作成される。化学修飾手段としては表面グラフト法とラジカル重合の2つの方法を用いることができる。またコーティング方法としては、適用温度範囲内で不溶とした後、不溶なものコーティングする。これらを図示すると、図1の通りである。本発明のクロマトグラフィー担体の製造方法の具体的手段の一例として次の化学式を参照して述べる。

【0013】

【化3】



【0014】N－イソプロピルアクリルアミドモノマー（1）、2，2'－アゾビス（イソブチロニトリル）（AIBNと略記）、3－メルカプトプロピオン酸（MPAと略記）をN，N－ジメチルホルムアミド溶媒に溶かし、液体窒素を用いて凍結脱気をした後、70±1℃においてテロメリゼーションによって重合した。これを濃縮し、ジエチルエーテルによって沈澱させ片末端にカルボキシル基を持ったポリ（N－イソプロピルアクリル※アミド）（2）を得る。粗生成物は溶解再沈法で精製する。これをシリカゲルをいれたデシケーター中に入れ、常温減圧下にて乾燥する。これを乾燥酢酸エチルに溶解し、ジシクロヘキシルカルボジイミド（DCCと略記）、N－ヒドロキシこはく酸イミドを加え室温で反応させポリ（N－イソプロピルアクリルアミド）のカルボキシル基を活性エステル化した後、濃縮してジエチルエーテル中に滴下して沈澱させる。次に常温減圧乾燥し、

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活性エステル化ポリ（Ｎ－イソプロピルアクリルアミド）（３）を得る。これを純水に溶かしアミノ基含有担体を加え反応してアミド結合を形成することによりポリ（Ｎ－イソプロピルアクリルアミド）を担体にグラフト、コーティングしたもの（４）を得る。本発明における担体を使用して分離・精製できるものとしては生理活性を有するタンパク質や細胞などで、具体的に牛血清アルブミン、ＩｇＧ、フィブリノーゲン、フィブロネクチン、トランスフェリン、血液凝固因子等を挙げることができる。

【００１５】

【実施例】次に実施例をもって、具体的に本発明を説明する。

実施例１

（ａ）片末端にカルボキシル基を有するポリ（Ｎ－イソプロピルアクリルアミド）の合成法

Ｎ－イソプロピルアクリルアミド２０．０ｇ、３－メルカプトプロピオン酸０．１９ｇ、２，２’－アゾビス（イソブチロニトリル）０．２１ｇをそれぞれ重合管にいれ、乾燥Ｎ，Ｎ－ジメチルホルムアミド５０ｍｌを加えて溶解した。次に液体窒素下で凍結した後真空オイルポンプで重合管中の酸素を脱気し、減圧状態のまま重合管をメタノールに浸しＮ，Ｎ－ジメチルホルムアミド中の溶存酸素を取り除いた。この凍結脱気の操作を３回繰り返し行った。脱気が完全にできたら７０±１℃のインキュベーターで１７時間反応させた。次に、室温まで下がったら減圧濃縮を行う乾燥ジエチルエーテル中に滴下させ片末端にカルボキシル基を持ったポリ（Ｎ－イソプロピルアクリルアミド）を沈澱させた。この沈澱物をＰＴＦＥ（ポリテトラフルオロエチレン）フィルター（ポアサイズ３．０μｍ）で濾取し、シリカゲルを入れたデシケーター中で減圧乾燥をし、粗生成物１８．０ｇが得られた。これを乾燥Ｎ，Ｎ’－ジメチルホルムアミド３０ｍｌに溶かした後、乾燥ジエチルエーテル中に滴下し、その沈澱物をテフロンフィルターで濾取した。これをデシケーター中で減圧乾燥をおこない精製ポリ（Ｎ－イソプロピルアクリルアミド）を得た。Ｎ－イソプロピルアクリルアミド８．０ｇ、Ｎ，Ｎ－ジメチルアクリルアミド２．０ｇ、３－メルカプトプロピオン酸０．１８ｇ、Ｎ，Ｎ’－アゾビスイソブチロニトリル０．１ｇを精製したＮ，Ｎ－ジメチルアクリルアミド５０ｍｌに溶解し、上記と同様に脱気封管後７０±１℃で１２時間重合した。上記と同様の再沈精製を行い、片末端にカルボキシル基を有するＮ－イソプロピルアクリルアミド共重合体を得た。得られた共重合体は水溶液中で４３℃付近で相転移を示した。合成の仕込み等に、Ｎ－イソプロピルアクリルアミドモノマーに対するＮ，Ｎ－ジメチルアクリルアミドモノマーの量を変化させることによって任意の温度で相転移を示す共重合体が得られる。得られた各ポリマーはテトラヒドフランを溶媒としたゲル濾過クロマトグラフィー及び酸－塩基測定によりポリ（Ｎ－イソプロピルアクリルアミド）が分子量１０，０００、Ｎ－イソプロピルアクリルアミド－Ｎ，Ｎ－ジメチルアクリルアミド共重合体が分子量８，０００であり、各分子末端に約１個のカルボキシル基を有することを確認した。

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【００１６】（ｂ）片末端にカルボキシル基を有するポリ（Ｎ－イソプロピルアクリルアミド）の活性エステル化

精製ポリ（Ｎ－イソプロピルアクリルアミド）を１１．３５ｇを乾燥酢酸エチル１００ｍｌ中に溶かし、ジシクロヘキシルカルボジイミド１．２３ｇ及びＮ－ヒドロキシこはく酸イミド０．６９ｇを加えてよく撹拌しながら０℃で２時間、室温（２０～２５℃）で１２時間反応させた。次に、副反応物であるＮ，Ｎ’－ジシクロヘキシル尿素をＰＴＦＥフィルターで濾取し、その濾液を減圧濃縮した後乾燥ジエチルエーテル中に滴下し沈澱したものをテフロンフィルターで濾取して、常温減圧で溶媒を留去したものについて、活性エステル化ポリ（Ｎ－イソプロピルアクリルアミド）を得た。片末端カルボン酸Ｎ－イソプロピルアクリルアミド－Ｎ，Ｎ－ジメチルアクリルアミド共重合体も同様にして、活性エステル化した。

【００１７】（ｃ）活性エステル化ポリ（Ｎ－イソプロピルアクリルアミド）とアミノ基担体との結合

活性エステル化ポリ（Ｎ－イソプロピルアクリルアミド）２．０ｇを純水５０ｍｌに溶かし、アミノプロピルシリカゲル６．０ｇを加え、１２時間室温で激しく振とうして反応させた後冷水５００ｍｌで洗浄し、再び活性エステル化ポリ（Ｎ－イソプロピルアクリルアミド）２．０ｇを純水５０ｍｌに溶かした溶液中に加え、１２時間室温で激しく振とうした。この操作を３回繰り返し、冷水５００ｍｌで洗浄した後、メタノール１００ｍｌで洗浄し、乾燥した。活性エステル化ポリ（Ｎ－イソプロピルアクリルアミド）３．０ｇを６ｍｌのＮ，Ｎ－ジメチルホルミアミドに溶解し、これを表面に一級アミノ基を導入したポリスチレン微粒子浮遊液１ｍｌ（直径１．０±０．０３μｍ、原液濃度：5×10^{11}個／ｍｌ）を２４ｍｌの純水で希釈した液に１ｍｌづつ３０分間隔で加え、ゆっくりと転倒混和した。全量を加えた後、４℃以下で１６時間転倒混和した。反応終了後、遠心分離による回収と冷純化による洗浄を２回繰り返した後、ハンクス平衡塩溶液（ｐＨ７．４）を用いて希釈した（6×10^{9}、6×10^{10}／ｍｌ）。

【００１８】次に本発明の担体を用いてクロマトグラフィーを行った例を示す。

実施例２

（ａ）空カラムへの充填（湿式スラリー充填法）

ポリ（Ｎ－イソプロピルアクリルアミド）修飾シリカゲル２．０ｇを純水１０ｍｌに懸濁し、予め空カラム

（4．6φ×150mm）につないであるパッカー内に注ぎ、直ちに蓋を締め圧力が350kg／cm²で2時間、300kg／cm²で3時間純水を送液して充填した。

（b）本発明による充填剤を用いたクロマトグラフィー分離例

上記のポリ（N－イソプロピルアクリルアミド）修飾シリカゲルを固定相としたカラムに医薬品のヒドロコルチゾン（1）と酢酸ヒドロコルチゾン（2）の混合物を試料として注入した場合の分離例を示す。ヒドロコルチゾン（1）と酢酸ヒドロコルチゾン（2）とを混合した試料を注入し、これを移動相として水を毎分1．0mlの割合で流し、紫外可視吸光度検出器（測定波長254nm）を用いて測定した。その結果を図2に示す。図2より5℃、15℃、30℃、50℃と温度をあげることにより、水のみの移動相で分離可能となったことが示される。図2はヒドロコルチゾン（1）と酢酸ヒドロコルチゾン（2）の温変化に伴う保持時間の変化を示した。図3－aは、ベースとなるアミノプロピルシリカゲル担体を充填剤として用いた場合であり、図3－bは本発明を用いた充填剤による分離の場合である。図3における温度の影響を明らかにするために、図4において、$\log k'$と1／Tの関係をプロットを示す。明らかにベースのシリカゲルや従来のクロマトグラフィーにおける分離とは、全く異なった保持挙動を示している。

【0019】（c）本発明による充填剤を用いたクロマトグラフィ分離例2

上記のポリー（N－イソプロピルアクリルアミド）修飾シリカゲルを固定相としたカラムにベンゼン（基準物質）および5種のステロイド医薬品との混合物を試料として注入した場合の分離例を示す。カラムにベンゼン（1）、ヒドロコルチゾン（2）、プレドニゾロン（3）、デキサメサゾン（4）、酢酸ヒドロコルチゾン（5）、テストステロン（6）の6種を混合した試料を注入し、これを水を移動相として毎分1．0mlの割合で流し、紫外可視吸光度検出器（波長254nm）を用いて測定した。その結果を図5に示す。図5において50℃では、15分以上であったテストステロンの溶出時間をカラム温度を5℃に変化させることにより、6分以内に短縮することができた。このように外部温度をコントロールすることにより自由に試料の溶出時間を変化させることが可能である。また、従来のクロマトグラフィーでは有機溶媒との混合液を移動相に用いなければ分離できなかった試料を5℃～50℃の適当な温度に変化させることにより水のみの移動相によって完全な分離を達成することができた。

【0020】（d）温度応答性高分子修飾表面とリンパ球との温度制御クロマトグラフィー

温度応答性N－イソプロピルアクリルアミドーN，N－ジメチルアクリルアミド共重合体（IPAAm－DMA，組成20％モル）をグラフトした微粒子をハンクス平衡塩溶液に浮遊させ、ガラスカラム（8φ×300mm）に高さ100mm程度湿式充填した。ラット腸間膜リンパ節由来のリンパ球浮遊液（3×10^{8}cell／ml）とポリマーグラフト微粒子浮遊液1ml（6×10^{10}個／ml）をハンクス平衡塩溶液にて湿潤させたカラム上部に積層した。このカラムを恒温槽中で40℃に安定させた後、以下の実験を行った。溶離液として40℃に保温したハンクス平衡塩溶液を用いた場合は、カラム下部からの溶出液中には、リンパ球の溶出は見られなかった。続いてカラムを恒温槽中で10℃に安定させた後、10℃のハンクス平衡塩溶液を溶離液として用いた時、リンパ球は100％溶出した。この溶出液中の生存率を0．2％ニグロシン溶液を用いて観察した結果、カラムから脱離後にリンパ球は100％生存していることが確認された。

【0021】

【発明の効果】以上のように、温度応答性高分子を担体表面に導入した充填剤を固定相として使用することで温度変化による固定相の表面特性の制御が可能となり、水中及び水系によって生理活性物質や生きた細胞の分離・回収やその間に動く相互作用の温度制御が実現され、その結果、単一の水系の移動相によってタンパク質や細胞などの生体要素の機能を維持したままで分離・回収が可能と成るので夾雑物の混入を防止することができた。

【図面の簡単な説明】

【図1】本発明の担体表面の説明図

【図2】ヒドロコルチゾン（1）と酢酸ヒドロコルチゾン（2）の溶離に及ぼす温度影響を示す。

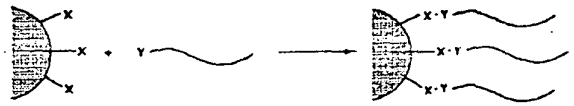
【図3】ヒドロコルチゾン（1）と酢酸ヒドロコルチゾン（2）の温度変化に伴う保持時間の変化を示す。a図は充填剤としてアミノプロピルシリカゲル、b図は本発明の充填剤である。

【図4】移動相として水を用いた場合、カラム中のヒドロコルチゾン（1）と酢酸ヒドロコルチゾン（2）に対するファント　ホッフプロット図を示す。a図は充填剤としてアミノプロピルシリカゲル、b図は本発明の充填剤である。

【図5】ベンゼン（1）、ヒドロコルチゾン（2）、プレドニゾロン（3）、デキサメサゾン（4）、酢酸ヒドロコルチゾン（5）、テストステロン（6）の溶離に及ぼす温度の影響を示す。

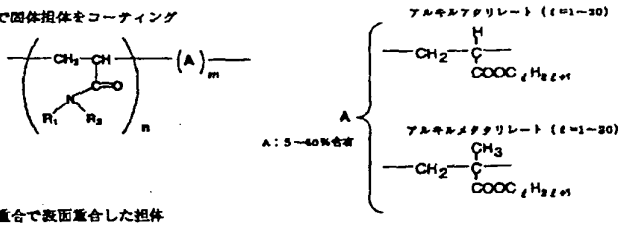
【図1】

反応式①．表面グラフト

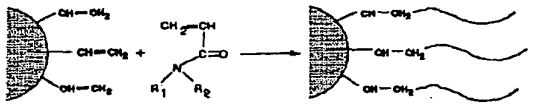


Y：片末端に官能基を持つポリ-アルキルアクリルアミド或はその共重合体
X:アミノ基、カルボキシル基、水酸基　　Y:カルボキシル基、アミノ基、水酸基

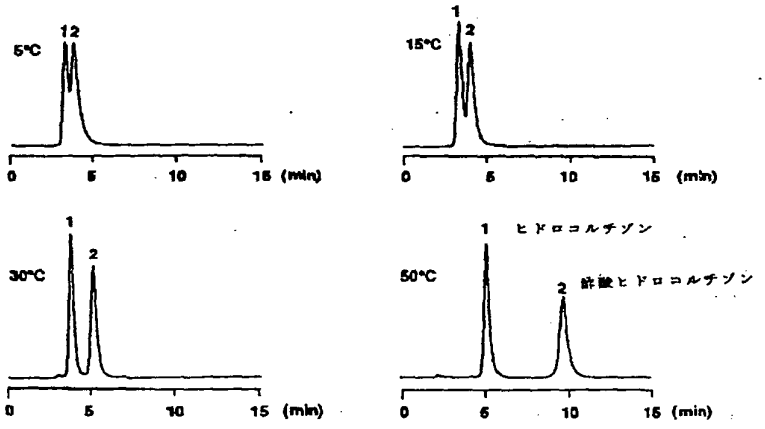
反応式②．共重合体で固体担体をコーティング



反応式③．ラジカル重合で表面重合した担体

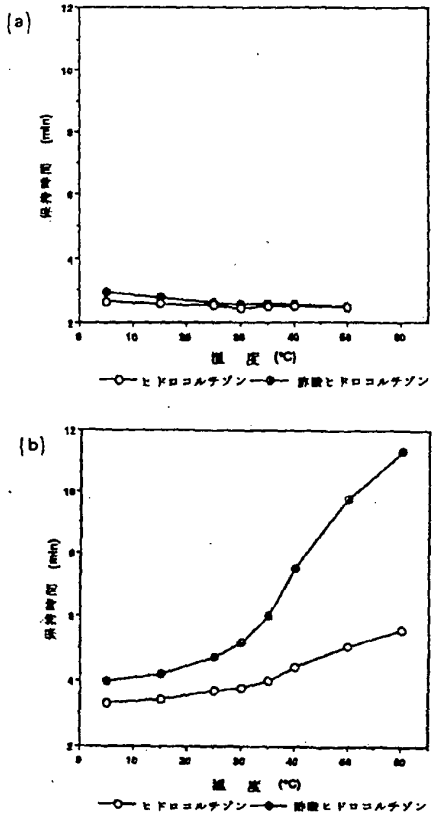


【図2】

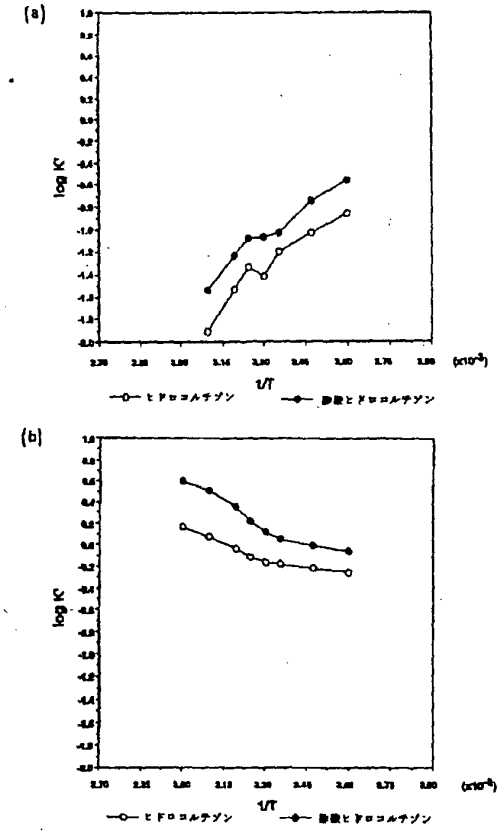


溶離液：H_2O.
流速　　：1.0ml/min.
測定器　：254nm(UV).
試料　：ヒドロコルチゾン，酢酸ヒドロコルチゾン

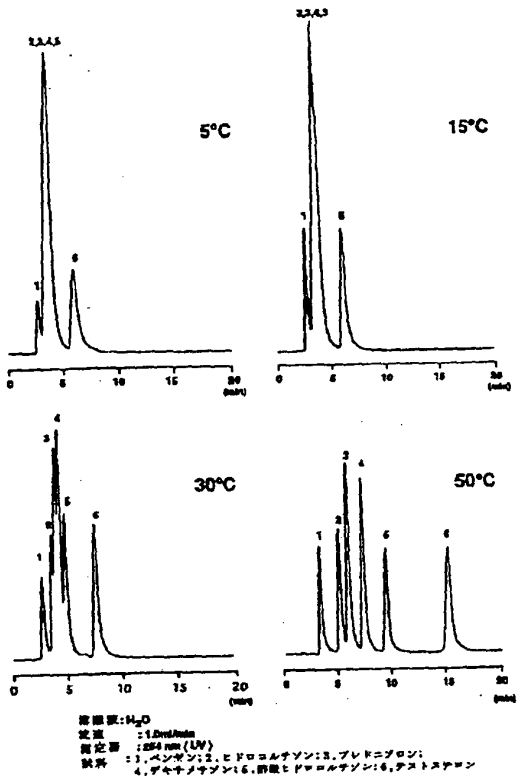
【図3】


(a)
保持時間 (min)
温 度 (℃)
ヒドロコルチゾン
酢酸ヒドロコルチゾン
(b)
保持時間 (min)
温 度 (℃)
ヒドロコルチゾン
酢酸ヒドロコルチゾン

【図4】


(a)
log K'
1/T
(x10⁻³)
ヒドロコルチゾン
酢酸ヒドロコルチゾン
(b)
log K'
1/T
(x10⁻³)
ヒドロコルチゾン
酢酸ヒドロコルチゾン

【図５】



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(54) CHROMATOGRAPHY AND FILLER THEREFOR

(57)Abstract:
PURPOSE: To realize separation and recovery while sustaining the function of biological element in the mobile phase of single water system by employing a filler wherein the balance of hydrophilicity and hydrophobicity on the surface of fixed phase can be varied depending on the temperature while fixing the mobile phase to the water system.
CONSTITUTION: The filler being employed in the separation of solute, e.g. a biological element or a cell, includes a filler where the balance of hydrophilicity and hydrophobicity on the surface of fixed phase can be varied by an external signal, e.g. temperature variation, while fixing the mobile phase to the water system, e.g. a filler where the surface of a carrier having amino group, carboxyl group, hydroxy group, etc., on the surface is chemically modified with polyalkyl acryl amide having an amino group at the end or a copolymer thereof. When such filler is employed, a biological element is adsobed to the hydrophobic surface upon reaching a critical temperature and it is separated or exfoliated as the temperature drops. Consequently, an organic solvent, a surfactant, etc., does not act as a dirt and the inventive method can be utilized in the separation, as well as analysis, while sustaining the function of protein or cell.

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[Date of final disposal for application]
[Patent number]
[Date of registration]
[Number of appeal against examiner's decision of rejection]
[Date of requesting appeal against examiner's decision of rejection]
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* NOTICES *

CLAIMS

[Claim(s)]
[Claim 1] The chromatography method characterized by separating a solute using the bulking agent which can change the hydrophilic property / hydrophobic balance on the front face of a stationary phase with an external signal, fixing a mobile phase to a drainage system.
[Claim 2] The chromatography method according to claim 1 that an external signal is a temperature change.
[Claim 3] The chromatography method according to claim 1 that a solute is a living body element or a cell.
[Claim 4] The chromatography method according to claim 1 which is the bulking agent for chromatographies with which the bulking agent carried out chemical modification of the carrier surface to the end by the poly alkyl acrylamide which has the amino group, a carboxyl group, or a hydroxyl group, or its copolymer.
[Claim 5] At an end to the support which has the amino group, a carboxyl group, or a hydroxyl group in a front face The amino group, After making a solute hold to the stationary phase which consists of a bulking agent for chromatographies which carried out chemical modification by the poly alkyl acrylamide which has a carboxyl group or a hydroxyl group, or its copolymer, The chromatography method characterized by separating a solute by changing the hydrophilic property / hydrophobic balance on the front face of a stationary phase by the step gradient method by the temperature gradient or temperature to which an outside temperature is changed gradually, and passing the same mobile phase.
[Claim 6] The chromatography method according to claim 5 that a mobile phase is a drainage system solvent.
[Claim 7] The bulking agent for chromatographies characterized by introducing a temperature responsibility macromolecule into a carrier surface.
[Claim 8] The bulking agent for chromatographies according to claim 7 whose temperature responsibility macromolecule is the poly alkyl acrylamide which has the amino group, a carboxyl group, or a hydroxyl group at the end.
[Claim 9] The bulking agent for chromatographies according to claim 8 whose poly alkyl acrylamide is any one sort of Polly (N-isopropyl acrylamide), the poly diethyl acrylamide, or poly acryloyl PIROIJIN.
[Claim 10] The bulking agent for chromatographies characterized by carrying out chemical modification of the poly alkyl acrylamide which has the amino group, a carboxyl group, or a hydroxyl group, or its copolymer to the carrier surface which has the amino group, a carboxyl group, or a hydroxyl group.

[Translation done.]

DETAILED DESCRIPTION

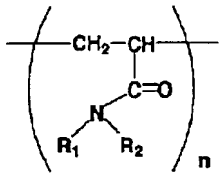
[Detailed Description of the Invention]
[0001]
[Industrial Application] this invention is a drainage system and it is related with the chromatography method that living body elements (protein, DNA, glycolipid, etc.) and a cell can be separated or refined using the bulking agent for chromatographies which can control the interaction of a solid-state front face and a cell membrane by the external signal (for example, temperature).
[0002]
[Description of the Prior Art] The combination of a high performance chromatography (HPLC) of a mobile-phase liquid and a stationary phase is various, and since many things can be chosen according to a sample, it is used for separation of matter various in recent years, and refining. A deer is carried out and the surface structure of a stationary phase is performed by changing the solvent of a mobile phase in the chromatography currently used conventionally in the interaction of the solute and stationary-phase front face which are included in the mobile phase, without making it change. for example, in HPLC currently used in many fields, the organic solvent which are methanol, an acetonitrile, etc. is used by the column of an antiphase system using the silica gel derivative which is using organic solvents, such as a hexane, an acetonitrile, and chloroform, as a mobile phase, and is separated by the drainage system as support in the column of a normal phase system using support, such as silica gel, as a stationary phase
[0003] Moreover, in the ion exchange chromatography which makes an anion exchanger or a cation exchanger a stationary phase, external ion concentration or a kind is changed and matter separation is performed. By rapid progress of genetic engineering etc., the use is broadly expected to various fields in which bioactive peptide, protein, DNA, etc. contain a drug, and its separation and refining have been a very important technical problem in recent years. Especially, separation and the need for technical to refine are increasing the physiological active substance, without spoiling the activity.
[0004] However, since the organic solvent and acid which are used for the conventional mobile phase, alkali, and a surfactant serve as impurity at the same time they spoil the activity of a physiological active substance, improvement of the system is expected. Moreover, separation / refining system which does not use these matter from the field of evasion of the environmental pollution of such matter is needed.
[0005]
[Problem(s) to be Solved by the Invention] As a result of examining many things that this invention persons should satisfy the above-mentioned request, the surface structure of a stationary phase then, for example, by changing external conditions, such as temperature It is what developed the technology separated and refined by changing the interaction on a solute and the front face of a stationary phase, without changing a mobile phase, and completed this invention. the purpose of this invention By changing external conditions, the surface characteristic of a stationary phase is changed in reversible, and the bulking agent as a stationary phase used for the chromatography method and this chromatography which can be separated and refined by the single drainage system mobile phase by this is offered.
[0006]
[Means for Solving the Problem] It is the chromatography method characterized by the summary of this invention separating a solute using the bulking agent which can change the hydrophilic property / hydrophobic balance on the front face of a stationary phase with an external signal, fixing a mobile phase to a drainage system. The carrier surface which has the amino group, a carboxyl group, or a hydroxyl group in a front face specifically It is the chromatography method using the bulking agent for chromatographies which carried out chemical modification to the end by the poly alkyl acrylamide which has the amino group, a carboxyl group, or a hydroxyl group, or its copolymer. That is, it becomes possible by using this invention to dissociate or exfoliate this by carrying out an outside temperature more than critical temperature by making living body elements, such as protein and a cell, stick to a hydrophobic front face, and reducing temperature. Therefore, since medicines, such as an organic solvent, an acid, alkali, and a surfactant, are not used at all in this case, it can use also for separation similarly to analysis, these preventing the contamination matter and a bird clapper, and maintaining functions, such as protein and a cell.
[0007] In the conventional chromatography method, when separating and analyzing two or more greatly different samples of the sample with which compounds various by one kind of mobile phase are mixed, especially polarity, separation is difficult and the time which separation takes will become very long. Therefore, although it is dissociating by the solvent gradient method or the step gradient method make it change gradually for changing the amount and kind of organic solvent continuously with time in case such a sample is treated It is possible to attain the same separation by changing column temperature continuously or gradually by the mobile phase single instead of using an organic solvent by the temperature gradient method or the step gradient method by this invention. By adopting this method, mixing of above-mentioned impurity is prevented, and while being able to dissociate with functions maintained, such as protein and a cell, a desired component is separable by controlling temperature in a short time.
[0008] The bulking agent for chromatographies used in this invention is a bulking agent which a temperature responsibility macromolecule is introduced into the front face, and can be changed with temperature changes by this by the hydrophilic property / hydrophobic balance on the front face of a bulking agent. That is, it is the bulking agent which carried out chemical modification by the poly alkyl acrylamide which is a temperature responsibility macromolecule about a carrier surface, and which has the amino group, a carboxyl group, or a hydroxyl group at the end, or its copolymer. As this bulking agent that carried out chemical modification, chemical modification of the

aforementioned poly alkyl acrylamide or its copolymer is carried out to the silica support which has functional groups, such as an amino group, a carboxyl group, or a hydroxyl group, on a front face, for example. And as silica support which has functional groups, such as an amino group, a carboxyl group, or a hydroxyl group, aminopropyl silica gel, amino sephadex, amino glass, ion exchange resin, etc. can be mentioned concretely. In this invention, any one sort of Polly (N-isopropyl acrylamide), the poly diethyl acrylamide, or the poly acryloyl pyrrolidine is desirable as a poly alkyl acrylamide. Therefore, it is as follows when the structure expression of the desirable poly alkyl acrylamide used in the invention in this application and its copolymer is shown.

[0009]

[Formula 1]

ポリーアルキルアクリルアミド

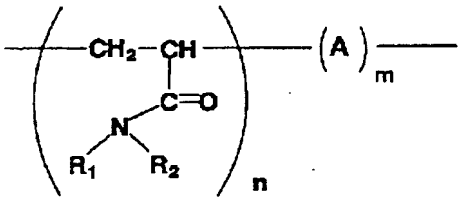


	R_1	R_2	Abbreviation
Poly(*N*-isopropylacrylamide)	—H	—CH(CH$_3$)$_2$	Poly(IPAAm)
Poly(*N*,*N*'-diethylacrylamide)	—C_2H_5	—C_2H_5	Poly(DEAAm)
Poly(acrylroylpyrolidine)			Poly(APy)

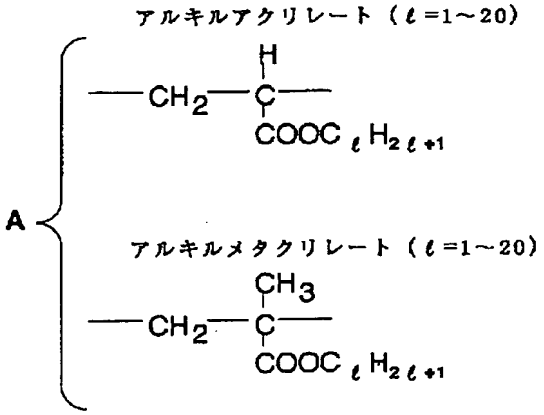
[0010]

[Formula 2]

共重合体



A：5～60％含有



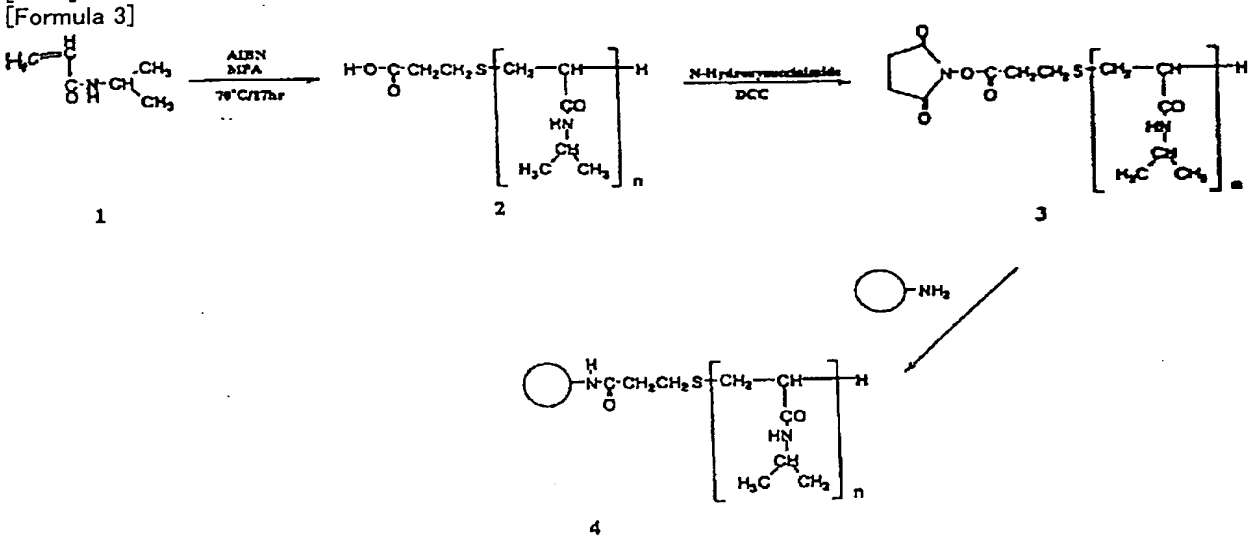
[0011] It becomes possible to control maintenance behavior with temperature, without changing composition of an eluate, since holding power [as opposed to / to the front face of the bulking agent of a chromatography / since, as for the support which carried out chemical modification by this molecule since poly (N-isopropyl acrylamide) had minimum critical temperature at 32 degrees C, surface physical properties change to hydrophilicity/canal with this critical temperature / a graft or when it is coated and used / a sample for this] changes with temperature. In order to make minimum critical temperature into 32 degrees C or more, it is possible to adjust by carrying out copolymerization of the acrylamide which is the monomer of a hydrophilic property, a methacrylic acid, an acrylic acid, a dimethyl

acrylamide, the vinyl pyrrolidone, etc. to an isopropyl acrylamide rather than an isopropyl acrylamide. Moreover, copolymerization with the styrene which is a hydrophobic monomer, alkyl methacrylate, alkyl acrylate, etc. can adjust to make minimum critical temperature into 32 degrees C or less.

[0012] Moreover, the minimum critical temperature of the poly diethyl acrylamide is about 30 degrees C - 32 degrees C, surface physical properties can change to hydrophilicity/canal bordering on this temperature, and the holding power to a sample can adjust it with temperature like the case where it is above-mentioned Polly (N-isopropyl acrylamide). The new support for chromatographies used by this invention is created by coating of chemical modification or a macromolecule. As a chemical modification means, the surface graft method and the two methods of a radical polymerization can be used. moreover, insoluble, as the coating method after being insoluble within an application temperature requirement — thing coating is carried out When these are illustrated, it is as drawing 1 . With reference to the following chemical formula, it states as an example of the concrete means of the manufacture method of the chromatography support of this invention.

[0013]

[Formula 3]



[0014] After melting N-isopropyl acrylamide monomer (1), 2, and 2'-azobis (isobutyronitrile) (azobisuisobutironitoriru and brief sketch) and 3-mercaptopropionic acid (MPA and brief sketch) to the N.N-dimethylformamide solvent and carrying out freeze deaeration using liquid nitrogen, in 70**1 degree C, the polymerization was carried out according to telomerization. This is condensed and poly (N-isopropyl acrylamide) (2) which was settled by diethylether and had a carboxyl group in the piece end is obtained. A rough product is refined by the dissolution reprecipitating method. This is put in into the desiccator into which silica gel was put, and it dries under ordinary temperature reduced pressure. After adding a dicyclohexylcarbodiimide (DCC and brief sketch) and an N-hydroxy amber acid imide, making it react at a room temperature and carrying out activity esterification of the poly (N-isopropyl acrylamide) carboxyl group, it condenses, and it is dropped into diethylether and made to dissolve this in dryness ethyl acetate and to precipitate. Next, ordinary temperature reduced pressure drying is carried out, and activity esterification poly (N-isopropyl acrylamide) (3) is obtained. A graft and the thing (4) with which it coated are obtained for poly (N-isopropyl acrylamide) to support by melting this to pure water, adding amino-group content support, reacting, and forming amide combination. It is protein, a cell, etc. which have physiological activity as separation and a thing which can be refined using the support in this invention, and a cow serum albumin, IgG, a fibrinogen, a fibronectin, a transferrin, a blood coagulation factor, etc. can be mentioned concretely.

[0015]

[Example] Next, it has an example and this invention is explained concretely.

Poly (N-isopropyl acrylamide) synthesis method N-isopropyl acrylamide 20.0g [which has a carboxyl group at the piece end of example 1(a)], 3-mercaptopropionic acidg [0.19] and 2, and 2'-azobis (isobutyronitrile) 0.21g was put into the polymerization pipe, respectively, and 50ml of dryness N.N-dimethylformamide was added, and it dissolved. Next, after freezing under liquid nitrogen, the oxygen in a polymerization pipe was deaerated by the vacuum oil pump, the polymerization pipe was dipped in methanol with the reduced pressure state, and the dissolved oxygen in N.N-dimethylformamide was removed. Operation of this freeze deaeration was repeated 3 times, and was performed. When deaeration was completely possible, it was made to react with a 70**1-degree C incubator for 17 hours. Next, when falling to the room temperature, poly (N-isopropyl acrylamide) which was made to trickle into dryness diethylether which performs vacuum concentration, and had a carboxyl group in the piece end was settled. These settlings were separated with the PTFE (polytetrafluoroethylene) filter (pore size 3.0micrometer), reduced pressure drying was carried out in the desiccator into which silica gel was put, and 18.0g of rough products was obtained. After melting this to Dryness N and N'-dimethylformamide 30ml, it was dropped into dryness diethylether and the settlings were separated by Teflon filter -. Reduced pressure drying was performed for this in the desiccator, and refining poly (N-isopropyl acrylamide) was obtained. It dissolved in N [which refined N-isopropyl acrylamide 8.0g, N, and N-dimethyl acrylamide 2.0g, 0.18g / of 3-mercaptopropionic acid /, N, and N'-azobisisobutyronitril 0.1g], and N-dimethyl acrylamide 50ml, and the polymerization was carried out at 70**1 degree C after the deaeration sealed tube like the above for 12 hours. The same reprecipitation refining as the above was performed, and N-isopropyl acrylamide copolymer which has a carboxyl group at the piece end was obtained. The obtained copolymer showed phase transition near 43 degree C in solution. The copolymer which shows phase transition at arbitrary temperature is obtained by changing the amount of N to N-isopropyl acrylamide monomer, and N-dimethyl acrylamide monomer to composite preparation etc. For each obtained polymer, poly (N-isopropyl acrylamide) is molecular weight 10,000 and

N-isopropyl acrylamide by the gel filtration chromatography and acid-base measurement which used the TETORAHIDO furan as the solvent. - N and N-dimethyl acrylamide copolymer is molecular weight 8,000, and it checked having about one carboxyl group at each molecule end.
[0016] (b) 11.35g is melted for poly (N-isopropyl acrylamide) activity esterification refining poly (N-isopropyl acrylamide) which has a carboxyl group at the piece end in 100ml of dryness ethyl acetate, and it was made to react at a room temperature (20-25 degrees C) by 0 degree C for 12 hours for 2 hours, adding dicyclohexylcarbodiimide 1.23g and 0.69g of N-hydroxy amber acid imides, and stirring. Next, activity esterification poly (N-isopropyl acrylamide) was obtained about what separated the N and N'-dicyclohexyl urea which is a side reaction object with the PTFE filter, separated what trickled into dryness diethylether and precipitated by Teflon filter - after carrying out vacuum concentration of the filtrate, and distilled off the solvent by ordinary temperature reduced pressure. Piece end carboxylic-acid N-isopropyl acrylamide - N and N-dimethyl acrylamide copolymer carried out activity esterification similarly.
[0017] (c) Avidity esterification poly (N-isopropyl acrylamide) 2.0g of activity esterification poly (N-isopropyl acrylamide) and amino-group support was melted to 50ml of pure water, aminopropyl silica gel 6.0g was added, 500ml of back cold water which shook violently at the room temperature for 12 hours, and was made to react washed, and it shook [be / under / solution / which melted activity esterification poly (N-isopropyl acrylamide) 2.0g to 50ml of pure water again] adding] violently at the room temperature After it repeated this operation 3 times and 500ml of cold water washed, it washed and dried by methanol 100ml. Activity esterification poly (N-isopropyl acrylamide) 3.0g was dissolved in 6ml N and N-dimethyl HORUMI amide, it added 1ml at a time to the liquid which diluted with 24ml pure water 1ml (the diameter of 1.0**0.03 micrometers, undiluted solution concentration:5x1011 piece/ml) of polystyrene particle suspension which introduced the first-class amino group into the front face for this at intervals of 30 minutes, and fall mixing was carried out slowly. After adding the whole quantity, fall mixing was carried out below 4 degrees C for 16 hours. After the reaction end, after repeating washing by recovery by centrifugal separation, and cold purification twice, it diluted using Hanks balanced salt solution (pH 7.4) (6x109 and 6x1010-/ml).
[0018] Next, the example which performed the chromatography using the support of this invention is shown.
Restoration to an example 2 (a) vacuum column (the wet slurry filling-up method)
Poly (N-isopropyl acrylamide) ornamentation silica gel 2.0g was suspended in 10ml of pure water, it poured in the packer beforehand connected with the vacuum column (4.6phix150mm), and the lid was fastened immediately, and the pressure sent the liquid by 350 kg/cm2 for 2 hours, sent pure water by 300 kg/cm2 for 3 hours, and it was filled up.
(b) The example of separation at the time of injecting the mixture of the hydrocortisone (1) of medical supplies and an acetic-acid hydrocortisone (2) into the column which made the stationary phase the poly (N-isopropyl acrylamide) ornamentation silica gel of the example above of chromatography separation using the bulking agent by this invention as a sample is shown. The sample which mixed the hydrocortisone (1) and the acetic-acid hydrocortisone (2) was poured in, water was poured at a rate of 1.0ml/m by having made this into the mobile phase, and it measured using the ultraviolet visible absorbance detector (measurement wavelength of 254nm). The result is shown in drawing 2 . Having become separable by the mobile phase of only water is shown by by raising 5 degrees C, 15 degrees C, 30 degrees C, 50 degrees C, and temperature from drawing 2 . Drawing 2 showed change of the holding time accompanying ****** of a hydrocortisone (1) and an acetic-acid hydrocortisone (2). Drawing 3 -a is the case where the aminopropyl silica gel support used as the base is used as a bulking agent, and drawing 3 -b is the case of separation by the bulking agent which used this invention. In order to clarify the temperature effects in drawing 3 , in drawing 4 , a plot is shown for the relation between 1ogk' and 1/T. The separation in the silica gel and the conventional chromatography of the base shows completely different maintenance behavior clearly.
[0019] (c) The example of separation at the time of injecting mixture with benzene (primary standard) and five sorts of steroid medical supplies into the column which made the stationary phase the Polly (N-isopropyl acrylamide) ornamentation silica gel of the example of chromatography separation 2 above using the bulking agent by this invention as a sample is shown. Benzene (1), a hydrocortisone (2), the prednisolone (3), the dexamethasone (4), the acetic-acid hydrocortisone (5), and the sample that mixed six sorts of a testosterone (6) were injected into the column, it was poured at a rate of 1.0ml/m, having used water as the mobile phase, and this was measured using the ultraviolet visible absorbance detector (wavelength of 254nm). The result is shown in drawing 5 . In drawing 5 , the elution time of the testosterone which was 15 minutes or more was able to be shortened within 6 minutes by changing column temperature to 5 degrees C at 50 degrees C. Thus, it is possible by controlling an outside temperature to change the elution time of a sample freely. Moreover, in the conventional chromatography, the mobile phase of only water was able to attain perfect separation by changing the sample which has not been separated if mixed liquor with an organic solvent was not used for a mobile phase to the suitable temperature of 5 degrees C - 50 degrees C.
[0020] (d) Temperature-control chromatography temperature responsibility N-isopropyl acrylamide of a temperature responsibility macromolecule ornamentation front face and a lymphocyte - The particle which carried out the graft of the N and N-dimethyl acrylamide copolymer (IPAAm-DMA, 20% mol of composition) was made to float in Hanks balance salting-in liquid, and wet restoration was carried out a height of about 100mm at the glass column (8phix300mm). The laminating of the lymphocyte suspension (3x108 cell/ml) of the rat mesenteric lymph node origin and the 1ml (6x1010 pieces/(ml)) of the polymer-graft particle suspension was carried out to the column upper part which carried out humidity with Hanks balance salting-in liquid. The following experiments were conducted after stabilizing this column at 40 degrees C in a thermostat. When the Hanks balance salting-in liquid which kept it warm at 40 degrees C as an eluate was used, elution of a lymphocyte was not seen in the eluate from the column lower part. Then, when 10-degree C Hanks balance salting-in liquid was used as an eluate after stabilizing a column at 10 degrees C in a thermostat, the lymphocyte was eluted 100%. As a result of observing the survival rate in this eluate using a Nigrosine solution 0.2%, it was checked after desorption from the column that the lymphocyte survives 100%.
[0021]
[Effect of the Invention] As mentioned above, it becomes controllable [the surface characteristic of the stationary phase by the temperature change] by using the bulking agent which introduced the temperature responsibility macromolecule into the carrier surface as a stationary phase. The temperature control of the interaction which moves by underwater and the drainage system between them [of a physiological active substance or the useful cell / separation and recovery, or between them] is realized. Consequently, since it changed that separation and recovery were possible, maintaining the function of living body elements, such as protein and a cell, by the mobile phase of a single drainage system, mixing of impurity was able to be prevented.

[Translation done.]

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]
[Drawing 1] Explanatory drawing of the carrier surface of this invention
[Drawing 2] The temperature influence affect the elution of a hydrocortisone (1) and the hydrocortisone acetate (2) is shown.
[Drawing 3] Change of the holding time accompanying the temperature change of a hydrocortisone (1) and an acetic-acid hydrocortisone (2) is shown. As a bulking agent, a view is aminopropyl silica gel and b view is the bulking agent of this invention.
[Drawing 4] FANTO to the hydrocortisone (1) and acetic-acid hydrocortisone (2) in a column when water is used as a mobile phase A HOFFU plot view is shown. As a bulking agent, a view is aminopropyl silica gel and b view is the bulking agent of this invention.
[Drawing 5] Benzene (1), a hydrocortisone (2), a prednisolone (3), a dexamethasone (4), an acetic-acid hydrocortisone (5), and the temperature effects that it has on the elution of a testosterone (6) are shown.

[Translation done.]